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Estimation of Incidence of Respiratory Syncytial Virus Infection in Schoolchildren Using Salivary Antibodies

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An assay for respiratory syncytial virus (RSV)–specific IgG in saliva is described. The assay was used to examine the incidence of RSV infection in schoolchildren 7–10 years old during one RSV season. One hundred and twenty-one volunteer children provided saliva samples in October 1997 and March 1998; 18% of the children showed a fourfold or greater rise in anti-RSV IgG in the second sample. This prevalence of antibody increase is similar to that described in previous studies that measured CFT levels in serum samples. Overall, the children who showed rises in antibody levels, indicating that they had experienced an RSV infection, had lower levels of RSV-specific antibody in their preseason samples than those who showed no increase ($P = 0.0018$). These results show that saliva is an adequate substitute for serum in some antibody tests and may be useful for community studies. Such studies may provide surrogate markers for susceptibility to infection, which should benefit the planning of vaccination strategies. ***J. Med. Virol. 61:81–84, 2000.***

KEY WORDS: respiratory syncytial virus; saliva; antibody levels

INTRODUCTION

Respiratory syncytial virus (RSV) is the major viral cause of lower respiratory tract illness in babies and infants. It is also an important cause of pneumonia in immunocompromised adults and the elderly [Han et al., 1999]. Individuals are susceptible to repeated infection with the virus: in infants the attack rate in a day care setting was found to range from 98% during the first epidemic to 75% for the second and 65% for the third [Henderson et al., 1979]. Estimates of the rate of reinfection of young children have ranged from 8% to 41% per epidemic; in adults the rate of reinfection is about 5% per epidemic [Monto and Lim, 1971; Cooney et al., 1975]. Use of virus isolation or antigen detection to determine infection rates is fraught with problems, owing to practical limitations of sampling during an acute episode. The Tecumseh study [Monto and Lim, 1971] used complement fixation tests (CFT) on serum samples taken at 6-month intervals, so that rises in titre could be detected. The necessity of taking blood samples makes this test difficult to implement on a large scale at the community level, particularly in children.

It is thought that the most common route of introduction of RSV into a family is through older siblings, since these siblings were found to be the most common index case [Hall et al., 1976]. Thus, studies of community transmission of RSV need to include school-age children, and the effectiveness of a potential vaccine also would need to examine this age group. Since several vaccines are nearing the clinical trials stage, noninvasive methods for examining the prevalence of RSV infection in the community are required.

Since the early studies on the epidemiologic characteristics of RSV, much more sensitive tests than CFT have been developed; antibody tests using saliva samples, rather than blood, are now feasible. The study reported here describes the development of an enzyme-linked immunosorbent assay (ELISA) for the titration of anti-RSV antibodies in saliva. The assay has two stages: first, the concentration of total IgG is determined, and then a standard amount of IgG is used in each anti-RSV ELISA. This assay was then used to estimate the incidence of RSV infection in schoolchildren from 7 to 10 years old.

MATERIALS AND METHODS

Subjects

Two junior schools with enrolments of children aged 7–11 years were involved in this study. Volunteer children in the first three forms (7–10 years) were recruited

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into the study; information was given to the parents, and signed consent was obtained from them in all cases. Demographic data were collected, but no record was kept of respiratory tract infections over the study period. Local ethical approval was given for the study.

Sample Collection

Saliva samples were collected during the course of the school day in October 1997 and March 1998. Salivettes (Sarstedt Ltd., Leicester, UK) were used for sample collection: these consist of a sterile cotton swab held in a double-chambered centrifuge tube so that the saliva can be retrieved by centrifugation of the collection tube. The use of the saliva collection device was explained to the children, who then provided the samples under the supervision of the research workers. The children were asked to wipe the swab around their gums so that samples would include crepuscular exudate as well as saliva. The samples were then immediately transported to the laboratory, the collection tubes were centrifuged, and the saliva was frozen at −20°C.

IgG Capture Assay

The saliva samples were thawed on ice, and an aliquot from each was diluted with an equal volume of 2× preservative buffer (20% foetal calf serum and 0.2% sodium azide in phosphate-buffered saline [PBS]). Flat-bottomed 96-well plates (Immulon 2; Dynex Technologies, Ashford, UK) were coated overnight at 37°C with 100 μl/well rabbit antihuman IgG (Dako Ltd., Cambridge, UK) diluted 1/3,000 in sodium carbonate coating buffer. The plates were washed four times in PBS containing 0.05% Tween 20 (PBS-T). The plates then were blocked with 5% dried milk powder in PBS-T (200 μl/well) at 37°C for 60 minutes. IgG calibrant standards (Binding Site, Edgbaston, England) (0.039–2.5 mg/l) were prepared by doubling dilution in PBS-T. Saliva was diluted 1/100 in PBS-T. Saliva samples were assayed in triplicate alongside the IgG standards in duplicate (10 μl/well in 40 μl /well blocking solution) by incubating at 37°C for 90 minutes. After the plates were washed, horseradish peroxidase–conjugated rabbit antihuman IgG (Dako) (1/1,000) was applied and incubated at 37°C for 2 hours. After washing, detection was performed using *O*-phenylenediamine as substrate, with the reaction being stopped with 2.5 mol/l sulphuric acid. Absorbances (OD) were read at 492 nm. Using data from the IgG standards, a calibration graph was drawn for each plate and used to calculate the total IgG concentration for each saliva sample.

Anti–Respiratory Syncytial Virus Enzyme-linked Immunosorbent Assay

RSV strain A2 was grown in BSC-1 cells until cytopathic effect was extensive. Mock and infected cell lysates were prepared by lysing the cells with 0.5% NP40 in water, followed by removal of cell debris by centrifugation. Ninety-six-well plates were coated overnight with either RSV-A2-infected or mock-infected cell lysates, diluted in 0.5% NP40; the lysates were allowed to dry onto the wells. The plates were fixed at room temperature for 10 minutes using 80% acetone in water, drained, and allowed to dry.

Paired saliva samples were assayed in triplicate against both RSV-A2 and mock lysates. Incubation times, wash conditions, and detection conditions were all as described for the IgG capture assay. Horseradish peroxidase–conjugated goat antihuman IgG (H+L) (Bio-Rad, Hercules, CA) was used as secondary antibody. Initially, saliva samples were screened at a single dilution corresponding to 1 mg/l IgG (50 μl /well), as determined by the IgG capture assay. Individuals showing an increase in RSV-specific OD between paired saliva samples were subjected to re-assay across a suitable dilution range, to determine any change in anti-RSV titre.

Titres were expressed as the minimum IgG concentration required to produce a positive anti-RSV response by ELISA; an OD of > 0.2 was considered positive, after subtraction of values given by mock-infected cell lysates. A fourfold or greater increase in titre was considered to be a significant change, indicative of RSV infection during the study period. The Mann-Whitney *U* test was used to compare October and March anti-RSV levels (as measured by OD using 1 mg/l IgG) and IgG levels for two groups—those that showed a significant increase in anti-RSV titre and those that showed no change. A two-tailed P value < 0.01 was considered statistically significant.

RESULTS

One hundred and twenty-one children provided saliva samples both in October 1997 and March 1998. Sixty-three children were boys (mean age, 8.0 years), and 58 were girls (mean age, 8.0 years). In general, the children found the use of salivettes acceptable and were enthusiastic about the study. The volumes recovered ranged from 15μl to 1.9 ml. Eight pairs of samples were excluded, owing to inadequate volume or very low IgG concentrations. Inadequate volume was due to the fact that a very small number of the younger children were reluctant to use the salivette as instructed. Low IgG concentration was probably the result of copious spitting into the tube.

The sample IgG concentrations were found to range between 3.8 mg/l and 165 mg/l. These values were used to dilute the saliva to give a constant IgG concentration for use in the preliminary anti-RSV ELISA. At 1 mg/l salivary IgG, 46 individuals showed a possible increase in anti-RSV response between samples; these samples were titrated fully to obtain accurate end-point values. For these individuals, the concentration of IgG that gave a positive signal in the RSV ELISA ranged from 0.25 mg/l to 16 mg/l salivary IgG.

Twenty children (18%) showed fourfold or greater increases in anti-RSV IgG titre. Another 27 children (24%) showed a twofold increase in titre, but this was not considered reliable data, since it could have been

TABLE I. Changes of Anti-RSV Titres in paired Saliva Samples

Change in anti-RSV titre	Number of children (%)	Mean anti-RSV IgG (OD) in October (range)[a]	Mean anti-RSV IgG (OD) in March (range)[a]
> Fourfold increase	20 (18%)	0.105 (0.014–0.382)	0.351 (0.047–1.23)
Twofold increase	27 (24%)	0.127 (0.025–0.323)	0.249 (0.046–0.682)
No change	57 (50%)	0.194 (0.015–0.611)	0.204 (0.015–0.728)
Reduction	9 (8%)	0.213 (0.084–0.357)	0.116 (0.032–0.166)
Excluded	8		
Total	121		

[a]OD given in anti-RSV IgG test using 1 mg/L IgG.

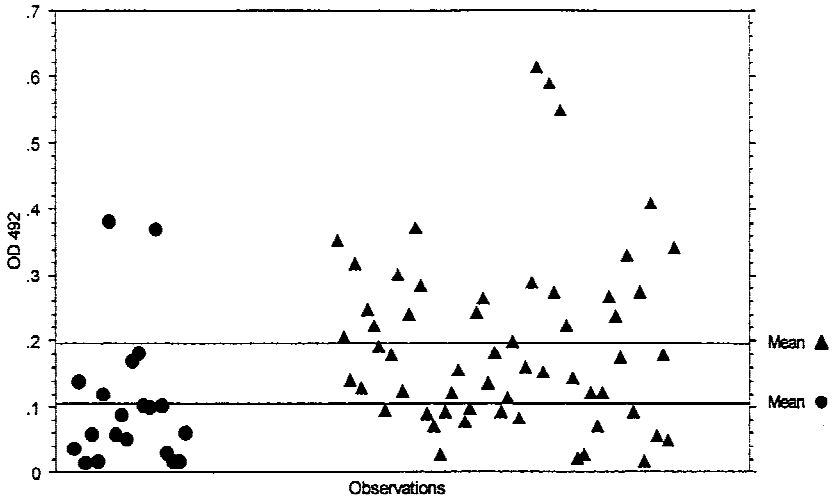

Fig. 1. A comparison of preseason anti-RSV IgG levels (as measured by OD in enzyme-linked immunosorbent assay with the IgG concentration at 1 mg/l) observed in two groups of children, those that subsequently showed a fourfold or greater rise in anti-RSV IgG (●) and those that showed no change (▲). The difference between the means was significant at $P = 0.0018$.

due to minor experimental variation. Fifty-seven children (50%) showed no change in titre, while the final nine children (8%) showed a reduction in titre, as determined by a decrease in OD in the initial screening assay. The changes in anti-RSV titre are summarised in Table I.

Statistical analysis was undertaken on data from two groups of children, comparing those that had a fourfold or greater rise in anti-RSV titre with those that had no change. The overall level of anti-RSV antibody (as indicated by OD obtained from anti-RSV ELISA using 1 mg/l total IgG) was significantly lower in the first sample in the group that subsequently showed a rise in titre compared with those that showed no change ($P = 0.0018$), as illustrated in Fig. 1. No significant difference was apparent between these groups for the second, post-RSV-season samples. In addition, neither of the groups showed any significant difference in IgG concentration in the specimens.

DISCUSSION

This report describes the development of an assay for anti-RSV antibodies in saliva and the application of the assay to estimate the incidence of RSV infection in schoolchildren. In this group of children, 7–10 years old, the incidence of increased anti-RSV titres during the winter of 1997–1998 was 18%. This figure is very similar to that obtained in the Tecumseh study using CFT data, where 19.7% of 5- to 9-year-olds and 16.9% of 10- to 14-year-olds showed rises in titre during 1 year of surveillance [Monto and Lim, 1971]. Thus, it would appear that measurement of titres of anti-RSV IgG in saliva is an adequate substitute for blood serum in serologic surveys and should be useful in providing an estimation of the rate of RSV infection. This study differs from many tests using saliva to estimate virus infection, in that most such tests use single samples only and look for the presence of IgM antibody. These results should be interpreted with caution, however, since this study did not determine by any other means, such as virus isolation, the actual incidence of RSV infection in the group studied.

Our findings are also compatible with the possibility that susceptibility of the children to natural RSV infection was related to their levels of anti-RSV antibody. It has been shown that immunisation of young children with an RSV F protein subunit vaccine caused substantial increases in neutralising antibodies, which were associated with significant protection against RSV infection [Tristram et al., 1994; Welliver et al., 1994]. Although the major impact of RSV is in young babies, RSV also is associated with exacerbations of asthma [Nicholson et al., 1993] and may be an important cause of death during the winter [Nicholson, 1996]. The test described here opens the way for far more extensive community studies of the transmission of RSV and

should allow baseline data to be obtained before the introduction of a potential vaccine.

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REFERENCES

Cooney MK, Fox JP, Hall CE. 1975. The Seattle virus watch. VI. Observations of infections with and illness due to parainfluenza, mumps, and respiratory syncytial viruses and *Mycoplasma pneumoniae*. Am J Epidemiol 101:532–551.

Hall CB, Geiman JM, Biggar R, Kotok DI, Hogan PM, Douglas GR. 1976. Respiratory syncytial virus infections within families. N Engl J Med 294:414–419.

Han LL, Alexander JP, Anderson LJ. 1999. Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J Infect Dis 179:25–30.

Henderson FW, Collier AM, Clyde WA, Denny FW. 1979. Respiratory-syncytial-virus infections, reinfections and immunity: a prospective longitudinal study in young children. N Engl J Med 300:530–534.

Monto AS, Lim SK. 1971. The Tecumseh study of respiratory illness. III. Incidence and periodicity of respiratory syncytial virus and *Mycoplasma pneumoniae* infections. Am J Epidemiol 94:290–301.

Nicholson KG. 1996. Impact of influenza and respiratory syncytial virus on mortality in England and Wales from January 1975 to December 1990. Epidemiol Infect 116:51–63.

Nicholson KG, Kent J, Ireland DC. 1993. Respiratory viruses and exacerbations of asthma in adults. Br Med J 307:982–986.

Tristram DA, Welliver RC, Hogerman DA, Hildreth SW, Paradiso P. 1994. Second-year surveillance of recipients of a respiratory syncytial virus (RSV) F protein subunit vaccine, PFP-1: evaluation of antibody persistence and possible disease enhancement. Vaccine 12:551–556.

Welliver RC, Tristram DA, Batt K, Sun M, Hogerman D, Hildreth S. 1994. Respiratory syncytial virus–specific cell-mediated immune responses after vaccination with a purified fusion protein subunit vaccine. J Infect Dis 170:425–428.